Review Article

Circulating biomarkers in epithelial ovarian cancer diagnosis: from present to future perspective

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Abstract: Ovarian cancer (OC) represents the most lethal gynecological cancer and the poor prognosis is often attributable to late diagnosis. The diagnostic approach to woman presenting with pelvic mass is difficult and differential diagnosis often requires invasive histological examination. Serum CA125 and HE4, as well as the most of the other serum biomarkers discovered and validated, are not sufficiently sensitive and specific to make early diagnosis. Moreover, conflicting results exist about the improvement of diagnostic performance by using multivariate index assays, developed by combining circulating biomarkers with other variables (i.e., ultrasound and/or menopausal status and/or age), in comparison to CA125 or HE4 alone. In the last years, several studies focused on the microRNAs (miRs), short single-stranded non-coding RNA that regulate several messenger RNAs (mRNAs). As in other cancer types, the aberrant miRs expression has been demonstrated in gynecological cancers, in both tissues and serum samples. In particular, the diagnostic performance of single or miRs panels resulted very high. However, to date, despite the potential clinical utility has been demonstrated, none of these miRs has been validated in large OC populations.

Keywords: Biomarkers; epigenetic biomarkers; HE4; microRNAs (miRs); ovarian cancer (OC)

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Introduction

Ovarian cancer (OC) represents the most lethal female reproductive tract malignancy worldwide (1).

Current strategies and methods for OC detection include pelvic examination and transvaginal ultrasonography (US), usually performed in symptomatic subjects (2). However, signs and symptoms are mostly nonspecific (i.e., dyspepsia, bloating, early satiety, gas pains, backache) and often evident only in advanced stages (3).

Additionally, it is often difficult to discriminate between malignant and benign ovarian masses and this differential diagnosis is frequently made only after invasive histological examination.

As a consequence, nearly 70% of patients are diagnosed with advanced disease (stage III or IV) with a 5-year survival rate lower than 30% (4). Contrarily, patient's prognosis is usually excellent if diagnosis is made in early stages.

From the discovery of CA125 in the early 80's (5), several studies have been performed to identify and validate new single biomarkers or panels of biomarkers with high clinical sensitivity and specificity in early stages.

Despite different approaches have been used for biomarker discovery and characterization, most successes were achieved thanks to progress in proteomics and to the development of high-throughput technologies (6).

The large group of biomarkers investigated includes different molecules as cytokines, acute phase reactants growth factors, proteases, hormones and coagulation factors. However, notwithstanding apparently encouraging

successes, a bridge between basic research and clinical practice (named translational oncology) (7,8) has not yet been built.

To date, only two markers [CA125 and Human Epididymis protein 4 (HE4)] have been approved by the FDA for monitoring treatment and detecting disease recurrence (9).

Since in the last decade another field of great interest has been epigenetics (DNA methylation, histone modifications and the expression of noncoding RNAs), several researchers tried to identify epigenetic biomarkers useful for OC detection. To date, no single biomarker displays high sensitivity and specificity to detect early OC and the implementation of a panel of epigenetic biomarkers is not yet feasible in clinical practice (10).

In the present review we provide an overview of the main characteristics of traditional biomarkers (i.e., CA125 and HE4) and we summarize the current knowledge regarding emerging epigenetic biomarkers [i.e., microRNAs (miRs)].

CA125

Currently, CA125, also known as mucin 16 or MUC16, a transmembrane glycoprotein produced by coelomic epithelium, is routinely used in the clinical practice and remains the only recommended serum marker to monitor the response to therapy and to confirm relapse (11,12).

However, several limits characterize this biomarker. Firstly, some OC histotypes do not release this mucin. In particular non-epithelial tumors (i.e., germ cells and sex cord-stromal tumors) do not constitutively express this glycoprotein, or only express low levels of the marker (13,14). Moreover, between epithelial OC, the release of CA125 is high in serous tumors, but lower in mucinous cancers (15). Accordingly, since also up to 20% of epithelial OC fail to express significant levels CA125 (16), it shows low sensitivity and specificity in early stages of disease. Conflicting results have been reported about the correlation between disease stage and preoperative serum concentrations of CA125: some studies (17-19) described a significant correlation between preoperative CA125 levels and International Federation of Gynecology and Obstetrics (FIGO) stage. On the other hand, other studies reported that CA125 expression is more strongly associated with OC subtype than with stage (20). Inconsistent results exist also about the preoperative and pre-chemotherapy prognostic and predictive value of serum CA125 concentrations (21-23).

CA125 displays a low specificity, by rising in several non-ovarian malignancies including cervix, breast, colon, pancreatic, lung, gastric and liver cancers (24,25). Increased CA125 levels are also observed in benign or malignant diseases affecting pleura, pericardium and peritoneum, that derive from coelomic epithelium (26) and in several pelvic diseases including endometriosis, ovarian cysts, pelvic inflammatory disease, myomas of the uterus and salpingitis, as well as non-gynecologic diseases including cirrhosis, ascites, peritoneal inflammation, pleuritis/pericarditis, pancreatitis, renal failure, liver disease (27). Since CA125 can also be expressed at the surface of inflammatory cells, high concentrations can be found in rheumatoid arthritis, scleroderma, lupus, and Sjögren's syndrome (28). Both pregnancy and menstrual cycle can modify CA125 levels (29,30) and cyclic combined hormone replacement therapy (HRT) might also be associated with increased levels of CA125 (31). Finally, Caucasian women show higher concentrations than African and Asian women (32).

HE4

The development of sensitive and fast protein microarray and high-throughput mass spectrometry (MS) techniques has led to the discovery of several candidate biomarkers highly expressed in OC compared to healthy controls (8,33). However, the specificity of candidate biomarkers must be successively assessed in the verification phase and finally both the sensitivity and specificity should be evaluated in a clinical setting (34).

Between the large number of proteins investigated in OC, the only really introduced in clinical practice is HE4 (9).

HE4 is a 13 KDa protein coded by the gene *WFDC2* which maps on human chromosome 20q12-13.1. Its mature glycosylate secretory form is approximately 25 KDa and consists of a single peptide and two whey acidic protein (WAP) domains containing a "four disulfide core" encompassing eight cysteine residues (35).

Schummer *et al.* demonstrated for the first time that HE4 gene was overexpressed in patients with ovarian carcinomas as compared with healthy controls (36).

A few years later, Hellström *et al.* measured HE4 concentrations in serum of patients with ovarian carcinoma and demonstrated that HE4 is a potentially useful biomarker for OC (37).

Several case-controls studies have been successively performed to evaluate the diagnostic performance of this biomarker by comparing it to that of the reference marker

CA125 (9,38-43).

The most of these studies demonstrate that HE4 is useful in the differential diagnosis of ovarian masses (44,45). Very interestingly, it has been recently demonstrated that overexpressed HE4 has a direct biological role in the promotion of OC cells proliferation, invasion and metastasis (46).

Consequently, this biomarker, inserted with CA125 in an algorithm called Risk for Ovarian Malignancy Algorithm (ROMA), has been cleared by the Food and Drug Administration (FDA) as a diagnostic tool in the OC diagnosis (47).

In *Table 1* we have summarized the main results of six meta-analysis comparing HE4 and CA125 as diagnostic markers for OC (48-53).

HE4 is not a perfect OC biomarker: it results increased also in other malignant neoplasms, especially of gynecologic (i.e., endometrial, tubal, vulvar cancer) and pulmonary origin (44,54-56). Accordingly, it has been proposed as a biomarker in other cancer types, in particular endometrial cancer (57-59) and lung cancer (56,60).

Moreover, many variables can affect the serum levels of this marker, as age (HE4 concentrations are reported higher in the elderly), smoke (higher by one-third in smokers than in non-smokers) and renal function (61).

Conflicting results have been reported about the relationship between HE4 values and menstrual cycle or hormonal treatment. While some authors observed higher HE4 concentrations in the ovulatory phase compared to that measured in the follicular and luteal phases (62), others affirmed that HE values are not dependent on menstrual cycle or hormonal treatment (63,64).

From single-marker diagnostics to multivariate index assays

Some multivariate index assays including the measure of one or more circulating biomarkers, have been developed as aid in the diagnostic approach to ovarian masses to determine the likelihood of malignancy (*Table 2*).

More than 25 years ago, Jacobs *et al.* proposed an algorithm, named "Risk of Malignancy Index" (RMI), by combining the values of CA125 with ultrasound and menopausal status (65). This algorithm has been used in three different versions (named RMI I, II, and III) for longtime in clinical practice in many countries (66,67).

Sensitivities and specificities for the prediction of OC among patients undergoing surgery for an adnexal mass resulted 78%, 79%, and 74%, and 87%, 81%, and 91%, for RMI I, II, and III respectively (65,68,69).

Skates *et al.* developed a few years later the longitudinal Bayesian "Risk of Ovarian Cancer algorithm" (ROCA) (70). This algorithm compares the CA125 profile of cases to that of HC and incorporates the known incidence of OC at a given age in calculating the risk. Accordingly, in ROCA mathematical model are included CA125 changes over time and the woman's age.

ROCA performance has been successively investigated in several multicenter trials (71-75). In all these investigations, the reported specificity was 99.8% and the positive predictive value was between 35.1% and 37.5%.

Ova1 is a 5-protein blood biomarker panel cleared in 2009 by the FDA for the triage of patients who have a pelvic mass and need to undergo surgery (76,77). Ova1 predicts low or high risk for OC by combining the second generation CA125-II with transferrin, beta-2 microglobulin, apolipoprotein A-1, and transthyretin. In a multicenter prospective trial involving 590 women scheduled for ovarian tumor resection, Ova1 score had 96% sensitivity, 35% specificity, 40% positive-predictive value (PPV), and 95% negative-predictive value (NPV) (76,78).

After the discovery in 2009 of HE4, Moore *et al.* developed the Risk of Ovarian Malignancy Algorithm (ROMA) for the prediction of epithelial OC in pre-operative triaging of women affected by pelvic masses (39). This algorithm includes HE4 concentrations, CA125 values and menopausal status and is validated by setting specificity at 75% and determining sensitivity. More in details, the logistic regression model includes coefficients for the natural log (NL) of both HE4 and CA125 values and is differently calculated according to menopausal status.

Conflicting results exist about diagnostic and predictive performance of ROMA. Although some authors observed that ROMA is better than RMI in the diagnosis of EOC (79) and that diagnostic accuracy is higher using the ROMA (AUC: 0.939; 95% CI: 0.902–0.977) than using HE4 (AUC: 0.930; 95% CI: 0.891–0.969) or CA125 (AUC: 0.902; 95% CI: 0.855–0.949) (80), our and other groups failed to demonstrate ROMA superiority over HE4 alone (48,81,82).

In the study of Karlsen *et al.*, performed on 1,218 patients with pelvic mass, the AUCs in differentiating benign from early stage OC were 0.854, 0.864, 0,897 and 0.905, for CA125, HE4, ROMA and RMI, respectively. The RMI performance (AUC: 0.945) was superior to that of CA125 (AUC: 0.925), HE4 (AUC: 0.905) and ROMA

Table 1 Main results of meta-analysis comparing HE4 and CA125 as diagnostic markers for OC

Reference	Sample size	Main results
(48)	N=2,878: 11 studies (until December 2011)	For differential diagnosis between EOC and BD (N=715 patients, 4 studies)
		Sensitivity:
		HE4: 0.79 (95% CI: 0.74–0.84)
		CA125: 0.77 (95% CI: 0.58–0.89)
		Specificity:
		HE4: 0.93 (95% CI: 0.87–0.96)
		CA125: 0.84 (95% CI: 0.76–0.90)
		AUC:
		HE4: 0.82 (95% CI: 0.78–0.85)
		CA125: 0.88 (95% CI: 0.85–0.91)
		For differential diagnosis between OC and BD (N=883 patients, 5 studies)
		Sensitivity:
		HE4: 0.77 (95% CI: 0.72–0.81)
		CA125: 0.73 (95% CI: 0.63–0.81)
		Specificity:
		HE4: 0.89 (95% CI: 0.82–0.93)
		CA125: 0.86 (95% CI: 0.81–0.90)
		AUC:
		HE4: 0.79 (95% CI: 0.76–0.83)
		CA125: 0.89 (95% CI: 0.85–0.91)
(49)	N=3,865: 18 studies (until November 2011)	For differential diagnosis between OC and BD
		Sensitivity:
		HE4: 0.74 (95% CI: 0.72–0.77)
		CA125: 0.76 (95% CI: 0.74–0.86)
		Specificity:
		HE4: 0.86 (95% CI: 0.84–0.87)
		CA125: 0.81 (95% CI: 0.73–0.89)
		AUC:
		HE4: 0.89
		CA125: 0.87
(50)	N=3,395: 11 studies (until June 2012)	For differential diagnosis between OC and BD
		Sensitivity:
		HE4: 0.74 (95% CI: 0.72–0.76)
		CA125: 0.80 (95% CI: 0.73–0.88)
		Specificity:
		HE4: 0.87 (95% CI: 0.85–0.89)
		CA125: 0.76 (95% CI: 0.74–0.85)
		AUC:
		HE4: 0.89
		CA125: 0.87

Table 1 *(continued)*

Table 1 *(continued)*

Reference	Sample size	Main results
(51)	N=3,653: 14 studies (until January 2012)	For differential diagnosis between OC and BD
		Sensitivity:
		HE4: 0.79 (95% CI: 0.76–0.81)
		CA125: 0.79 (95% CI: 0.77–0.82)
		Specificity:
		HE4: 0.93 (95% CI: 0.92–0.94)
		CA125: 0.78% (95% CI: 0.76–0.80)
(52)	N=4,729: 25 studies (until December 2012)	For differential diagnosis between OC and BD
		Sensitivity:
		HE4: 0.74 (95% CI: 0.72–0.76)
		CA125: 0.74 (95% CI: 0.72–0.76)
		Specificity:
		HE4: 0.90 (95% CI: 0.89–0.91)
		CA125: 0.83 (95% CI: 0.81–0.84)
		AUC:
		HE4: 0.89
		CA125: 0.85
(53)	N=7,640: 32 studies (until November 2013)	For differential diagnosis between OC and BD
		Sensitivity:
		HE4: 0.76 (95% CI: 0.72–0.80)
		CA125: 0.79 (95% CI: 0.74–0.84)
		Specificity:
		HE4: 0.93 (95% CI: 0.90–0.96)
		CA125: 0.82 (95% CI: 0.77–0.86)
		AUC:
		HE4: 0.89 (95% CI: 0.86–0.92)
		CA125: 0.87 (95% CI: 0.84–0.90)

BD, benign disease; CI, confidence interval; HE4, Human Epididymis protein 4; OC, ovarian cancer.

(AUC: 0.909) also in premenopausal women (83).

Lennox *et al.*, by comparing ROMA and RMI, concluded that both these scores have poor performance in early-stage disease and low sensitivity in non-serous histologic subtypes (84).

Recently, Yanaranop *et al.*, by enrolling in a longitudinal study 260 Thai women (n=74 affected by OC), observed that AUC in predicting OC was higher for RMI (0.876) than for ROMA, (0.862), CA125 (0.806) and HE4 (0.824). Moreover, they reported similar AUCs (0.844 and 0.856) for ROMA and RMI in premenopausal women, but RMI resulted superior to ROMA in postmenopausal women (AUCs: 0.879 and 0.840, respectively) (85).

In 2016, FDA cleared the next-generation of Ova1, named Overa, which includes CA125-II, HE4, apolipoprotein A-1, follicle stimulating hormone and transferrin (86). This new assay shows a high sensitivity but a low specificity: Ueland *et al.* reported a sensitivity of 94%, 89% and 91% with a specificity of 54%, 83% and 69%, for Ova1, ROMA and Overa, respectively (87).

Karlsen *et al.*, by enrolling 809 patients with benign ovarian disease and 246 women affected by OC, developed a biomarker-based index named Copenhagen Index (CPH-I) comprising HE4, CA125 and age (88). CPH-I was then validated in eight international studies comprising 1,060 patients with benign ovarian masses and 550 patients with

Table 2 Multivariate index assays for estimating the risk of OC in women presenting with pelvic mass

Multivariate index assays	Laboratory biomarkers	Imaging tools	Subject characteristics
RMI [1990]	CA125	Transvaginal ultrasound	Menopausal status
ROCA [1996]	CA125	None	Age
ROMA [2010]	CA125; HE4	None	Menopausal status
OVA1 [2011]	CA125-II; Transferrin; Beta-2 microglobulin; Apolipoprotein A-1; Transthyretin	None	None
CPH-I [2015]	CA125; HE4	None	Age
Overa [2016]	CA125-II; Transferrin; HE4; Apolipoprotein A-1; FSH	None	None

CPH-I, Copenhagen Index; FSH, Follicle stimulating hormone; HE4, Human Epididymis protein 4; RMI, Risk of Malignancy Index; ROCA, Risk of Ovarian Cancer algorithm; ROMA, Risk of Ovarian Malignancy Algorithm.

OC. Despite CPH-I performance resulted similar to that of ROMA and RMI (AUC: 0.960 *vs.* 0.954 and 0.959), this index has the advantage to be independent of ultrasound and menopausal status.

Accordingly, in a prospective study involving 1,218 women (n=252 OC), aimed to compare the performance of CA125, HE4, ROMA, CPH-I and RMI in the differentiation between benign and malignant ovarian tumor, the authors reported an AUC of 0.920 for CA125, 0.933 for HE4, 0.946 for ROMA, 0.959 for CPH-I and 0.958 for RMI (89).

However, when all ovarian malignant tumors (epithelial OC, borderline tumors, ovarian metastases and non-epithelial OC) are included in the analysis, the performances of both CPH-I and ROMA result significantly reduced (90).

Circulating miRs

miRs are active small (19–25 nt) non-coding highly conserved RNA molecules that bind specifically to, and post-transcriptionally regulate, several messenger RNAs (mRNAs) (91).

It has been demonstrated that miRs are involved in cancer biology through controlling expression of their target mRNAs to facilitate tumor growth, invasion, angiogenesis, and immune evasion (92).

The discovery that miRs circulate in the peripheral blood and can be thus measured not only invasively in tissues but also in plasma or serum, has open the possibility to use these nucleic acids as diagnostic and prognostic cancer biomarkers. Moreover, miRs have the advantage to be highly stable in plasma and serum and to be resistant to endogenous ribonuclease activity (93).

Accordingly, more than 20 studies have demonstrated that several miRs involved in different OC pathways are dysregulated (up- or down-expressed) in cancer patients (94-96).

The first study reporting the correspondence between over-expression of miRs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214) in OC tissue and serum-derived exosomes (97) was published in 2008.

One year later, Resnick *et al.* detected 21 miRs differentially expressed in serum of OC patients compared to healthy controls (96).

Different studies identified one or more miRs deregulated and thus potentially involved in OC carcinogenesis: Kan *et al.* demonstrated high expression levels of miR-200 family members (98), Xu *et al.* (99) found higher levels of miR-21, Guo *et al.* the up-regulation of miR-92 (100) and Zheng *et al.* observed the up-regulation of miR-205 and miR-483-5p and the down-regulation of let-7f in plasma of OC patients (101). In this last work let-7f and miR-205 were suggested as potential biomarkers for the early detection of EOC.

Chung and colleagues analyzed the RNA from two OC patients and a healthy control and demonstrated that 95 miRs were down-regulated and 88 miRs were up-regulated in the serum, tissue, and ascites of cancer patients (102). In the validation phase, performed by enrolling 18 patients and 12 controls, the authors observed that 5 miRNAs (miR-132, miR-26a, let-7b, miR-145, and miR-143) were markedly down-regulated in the serum from OC patients with respect to those of controls.

One year later, by using the deep sequencing technology (Solexa) and real-time PCR in serum samples of 31 OC patients, 23 patients with benign ovarian tumors and 8 control samples, Ji and colleagues demonstrated in cancer the up-regulation of miR-22, miR-93 and miR-451 and the down-regulation of miR-106b (103).

Zuberi *et al.* documented the high expression of miR-125b in serum of OC women and reported for miR-125b an AUC of 0.73 (95% CI: 0.64–0.81) to discriminate patients with malignant OC from healthy controls. At the best cut-off, the sensitivity and specificity were 62.3% and 77.1%, respectively (104).

By investigating 42 OC patients, 36 women diagnosed with a benign neoplasm and 23 healthy controls, Shapira *et al.* defined a 22-miRs profile to distinguish between OC and healthy controls and a 6-miRs profile to distinguish benign and OC patients (105).

Meng *et al.*, by enrolling 60 EOC patients and 20 affected by benign diseases, demonstrated that the combination of miR-200a, miR-200b and miR-200c displayed a sensitivity of 83% and a specificity of 100%, to differentiate malignant from benign ovarian tumors (106).

To explore in more depth the current knowledge of miRs as potential biomarkers for OC, we suggest to read three recent reviews on this topic (107-109).

Conclusions

In the past years, a wide spectrum of serological biomarkers for OC detection has been investigated. However, a perfect and reliable biomarker (stable, highly specific and sensitive, inexpensive), is currently unavailable.

Unfortunately, despite the potential clinical utility of the proposed circulating miRs, the most of these have been validated in small cohort sizes, and at present, HE4 and CA125 remain the only biomarkers approved and applied in clinical setting.

Finally, further investigations are needed to verify diagnostic performance and to validate in large populations multi-marker panels.

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Footnote

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